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# **Licking microstructure and hedonic changes after flavour preference learning in rats**

John Edmond Riordan & Dominic Michael Dwyer

*School of Psychology, Cardiff University*

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*May 2019, revision submitted to QJEP*

Address for correspondence:

School of Psychology

Cardiff University

Tower Building, Park Place

Cardiff

CF10 3AT, UK

Email: DwyerDM@cardiff.ac.uk

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### Abstract

Pairing a neutral flavour CS with a nutrient reward will create a learned preference for that CS. Prior studies suggest that this is accompanied by an increase in the hedonic value of the CS, although the reliability of this effect is yet to be fully established. Here, flavour CS+s were mixed with either 16% sucrose or maltodextrin (with control CS-s mixed with 2% solutions of the same carbohydrate). While a reliable preference for the CS+ was seen in every case, and there was a learned increase in lick cluster size when all conditions were considered together, this difference was significant in only one experimental condition considered alone. A meta-analysis of these results and similar published licking microstructure analysis studies found that the Cohen's  $d_{av}$  effect size for changes in licking microstructure after flavour preference learning was 0.16. This is far smaller than the effect sizes reported when assessing learned hedonic changes in flavour preference based on other test or training methods. Although this confirms that flavour preference learning produces hedonic changes in the cue flavours, the analysis of licking microstructure with training based on voluntary consumption of CS and US compounds may be an insensitive means of assessing such effects.

## Introduction

Food learning, in terms of the ability to remember which foods are nutritious and safe to eat – and conversely which are unsafe and may cause harm – is essential to humans and other animals. This ability has been studied via flavour preference and aversion learning. Both use methods broadly similar to classical conditioning studies and involve pairing a flavour conditioned stimulus (CS) with an unconditioned stimulus (US). Flavour preference learning studies typically use a nutritious and/or palatable solution as their US (Sclafani, 1991), while conditioned taste aversion studies typically use a substance that causes nausea or other physiological disturbance as the US (Lin, Arthurs, & Reilly, 2014; Parker, 2003). While both have received substantial attention, it remains the case that the analysis of flavour preference learning is underdeveloped relative to the analysis of learned taste aversions. Despite this, flavour preference learning is arguably the more practically important of the two given that the modal number of reported taste aversions per person is either one or zero (Rozin & Vollmecke, 1986) which is clearly insufficient to explain the richness of food choice.

One possible reason that taste aversion has received so much attention is that it has particularly dramatic effects that potentially reflect ‘special’ mechanisms not seen in other forms of learning. For example, the classic demonstration of selective aversion learning (Garcia & Koelling, 1966) showed that flavour-nausea pairings were more effective than audio-nausea pairings (and audio-pain pairings more effective than flavour-pain pairings). In addition, otherwise palatable flavours paired with nausea come to elicit reactions consistent with unpleasant bitter tastes (Pelchat, Grill, Rozin, & Jacobs, 1983). While there remains debate over the exact mechanisms underpinning taste aversion learning, and in particular whether the difference from other forms of learning is qualitative or quantitative, the importance of learned changes in palatability in understanding taste aversion learning is

widely accepted (Dwyer, Gasalla, Bura, & Lopez, 2017; Gasalla, Soto, Dwyer, & Lopez, 2017; Lin, Arthurs, & Reilly, 2017; Parker, 2003). In contrast, these critical issues of selectivity in learning and the involvement of learned changes in flavour palatability have received only limited attention in the study of preference learning. There is no experimental evidence directly assessing cue selectivity in preference learning – although the success of Pavlovian conditioning with auditory cues and food rewards (Mackintosh, 1974), would suggest that selectivity in preference learning is unlikely to be complete. Furthermore, there are only a very few studies directly examining whether flavour preference learning can induce hedonic change (In rats - Breslin, Davidson, & Grill, 1990; Dwyer, Pincham, Thein, & Harris, 2009; Forestell & LoLordo, 2003; Myers & Sclafani, 2001a, 2001b; Myers & Sclafani, 2003; In mice- Austen & Sanderson, 2016; Austen, Sprengel, & Sanderson, 2017).

While consumption changes may be linked to palatability changes, intake measures alone are non-specific and can also reflect other influences including the level of motivation (Berridge, 1996). In order to directly assess flavour palatability more specific indicators must be used – the two most common methods are the taste reactivity test and licking microstructure analysis. The taste reactivity test involves monitoring rats' orofacial responses to a flavour, typically infused via an oral cannula. Rats that are exposed to palatable flavours exhibit appetitive reactions such as tongue protrusions and rhythmic mouth movements, whereas those given unpalatable flavours will gape, rub their chins, and flail with their forelimbs (L. A. Parker, 2014). Notably, pairing a palatable flavour with an aversive outcome (the most usual example being nausea induced by lithium chloride) will reliably change the naturally appetitive responses to aversive ones (e.g., Berridge, Grill, & Norgren, 1981; Breslin, Spector, & Grill, 1992; Dwyer et al., 2017; Pelchat et al., 1983; for reviews see, Lin et al., 2017; Linda A. Parker, 2014). While the taste reactivity test typically involves a surgical procedure and an extended process of behavioural scoring, the fact that these

responses are translatable across species highlights the validity of this measure of hedonic responses (Berridge, 2000).

In contrast, licking microstructure analysis takes advantage of the behaviour of rodents as they drink naturally. Rats tend to drink in bursts of licks followed by short pauses, and the average size of these lick clusters has been shown to increase monotonically with increasing concentrations of palatable flavours (Davis & Smith, 1992), and to decrease with increasing concentrations of unpalatable tastes (Spector & St John, 1998). Importantly, while cluster size continues to increase with increases in the concentration of a palatable flavour, consumption reaches a peak and then begins to reduce (Ernits & Corbit, 1973), showing that cluster size is not simply determined by overall intake (Dwyer, 2012). Similar to the taste reactivity test, mean lick cluster size has reliably been shown to decrease when flavours are paired with aversive USs such as lithium chloride (e.g., Arthurs, Lin, Amodeo, & Reilly, 2012; Baird, St John, & Nguyen, 2005; Dwyer, Boakes, & Hayward, 2008; Dwyer, Gasalla, & Lopez, 2013; for reviews see, Dwyer, 2012; Lin et al., 2017).

These methods have been used to suggest that pairing a flavour with carbohydrates will increase appetitive taste reactivity responses to that flavour compared to a flavour paired with water (Breslin et al., 1990; Forestell & LoLordo, 2003; Myers & Sclafani, 2001b), and also produce a learned increase in lick cluster size (Dwyer, 2008; Dwyer et al., 2009; Myers & Sclafani, 2001a). Although these initial studies are encouraging, they are few in number, use a range of different conditioning procedures, involve relatively small numbers of animals, and produce a wide range of effect sizes. Moreover, there are also some contrasting results: Myers and Sclafani (2003) report changes conditioned preference but no changes in taste reactivity responses when mildly aversive CSs were paired with intragastric nutrient infusions; and reports of lick analysis following flavour preference conditioning in mice have produced inconsistent results (Austen & Sanderson, 2016; Austen et al., 2017). Considering

the importance of the analysis of palatability change to the investigation of the mechanisms for flavour learning, the current series of studies was designed to use licking microstructure analysis to confirm prior reports that flavour preference learning changes the palatability of cues and to estimate the degree of such a change. This series of studies also included auditory cues to investigate selectivity in flavour preference learning. Thus, in both Experiments 1 and 2, either 16% and 2% sucrose or 16% and 2% maltodextrin were paired with one of two Kool-Aid flavours or one of two different auditory stimuli. Unfortunately, there was no evidence of selective learning with the auditory stimuli in any experiment, with the suggestion of strong generalisation between the stimuli used. Therefore, this report will focus on the use of lick analysis to assess potential changes in palatability produced by flavour preference learning.

## Methods

---- Table 1 about here ----

Table 1 outlines the general designs for Experiments 1A, 1B, and 2. In each experiment one Kool Aid flavour (the CS+) was mixed with a 16% carbohydrate solution and a second flavour (the CS-) mixed with a 2% solution of the same carbohydrate (to ensure that it was consumed throughout training). Sucrose and maltodextrin were chosen as the reinforcing solutions due to their proven efficacy in supporting nutrient based learning (Ackroff & Sclafani, 1994; Bonacchi, Ackroff, & Sclafani, 2008; Sclafani, 2002; Sclafani, Cardieri, Tucker, Blusk, & Ackroff, 1993) and prior reports of producing conditioned changes in hedonic reactions to the CS (Breslin et al., 1990; Dwyer, 2008; Dwyer et al.,

2009). The concentrations chosen were 2% and 16% in order to create a strong preference - higher than 16% carbohydrate concentrations tend to produce smaller preferences (Lucas, Azzara, & Sclafani, 1997). During 1-bottle testing both CS+ and CS- were presented with 16% carbohydrate, both to avoid a negative contrast effect produced by experiencing the CS+ with a low concentration of the US and to minimise differences in consumption between the CS+ and CS- (Harris & Thein, 2005; Sclafani, 2002) without negatively impacting on lick cluster assessment (Dwyer, 2008). In the 2-bottle tests both were presented with 2% carbohydrate to try to stop rats solely consuming the first solution they tried. As previously mentioned, the experiments were designed to also assess cue selectivity in flavour preference learning. While this has not been described in detail, all experiments also included an audio group in which both the CS+ and CS- were auditory stimuli rather than flavours, but was otherwise identical to the flavour group.

### ***Experiment 1A***

#### *Subjects*

16 Lister-Hooded rats (Charles River, UK) were used, aged approximately 8 weeks on arrival and maintained on a food restricted diet at ~90% of their free-feeding weight (range, 290-330g). *Ad lib.* water was available throughout the experiment except for the pre-training phase, during which rats were given access to water for 1 hour a day after experimental sessions were completed. All rats were housed in groups of 4 and kept on a 12:12 hour light-dark cycle, with lights switched on at 7:30 am. Food rations were given roughly 1 hour after training sessions and experimental sessions began at approximately 10am.

#### *Apparatus*

All experimental sessions took place in four operant boxes (Med Associates, St Albans) measuring 30 × 24 × 21 cm. These boxes had a floor consisting of 19 steel rods, two walls made of aluminium and two walls and top made of clear plastic. One aluminium wall



contained two 1 cm diameter holes; one on the left and one on the right, each 5 cm from the respective wall and from the floor of the box. These holes allowed for drinking bottles consisting of a steel spout and 30 ml bottle to be accessed by rats inside the box, while the bottles were in the forward position. Bottles were automatically advanced/withdrawn at the beginning and end of each session. Licks were recorded by a computer running MEDPC (Med Associates, St Albans), which measured contact with a bottle spout to the nearest 0.01 second. A weighing scale accurate to 0.1 g was used to measure consumption by recording the weight of each bottle before and after each session. The solutions used in this experiment were 2% and 16% (w/w) sucrose made with deionised water. Flavours CSs were grape and cherry Kool-Aid (Kraft Foods USA, Rye Brook, NY) at 0.05% w/w.

### ***Procedure***

#### *Pretraining & Training*

Pretraining consisted of the rats being given water without any accompanying stimuli in the testing chambers for 20 minutes. This continued until all the rats were showing reliable consumption of at least 2 ml per session - which took 4 sessions.

The training phase consisted of 8 sessions, all 20 minutes long. Half of the animals had grape as the CS+ and cherry as the CS- (with the remainder receiving the reverse assignment). Half of each group received the CS+ on the first session (with the remainder receiving the CS-), with the CS+ and CS- presented four times in an ABBA order, alternating sides every day.

#### *Test*

Testing involved two phases: 1-bottle testing was first, with all rats receiving the CS+ and CS- separately, once each over two days. Each CS were presented with 16% sucrose (w/w), and half of the rats received the CS+ on the first day, with the other half receiving the CS-. Only the left-hand bottle was used for this phase. The second phase consisted of a 2-

bottle preference test in which animals were given simultaneous access to both the CS+ and the CS-, with both paired with 2% sucrose (w/w). Half of each group had their CS+ on the left side, and half on the right side. Test sessions lasted 20 minutes each.

### ***Experiment 1B***

#### *Subjects*

16 Lister-Hooded rats (previously used in the failed audio condition in Experiment 1A) were used, now aged approximately 16 weeks and weighing 267-296g. All were housed and fed under the same conditions as in Experiment 1A.

#### *Materials & Procedure*

The same materials were used as in Experiment 1A, with the exception that the US was maltodextrin (C\*Dry MD 01904, Cerestar-UK, Manchester, U.K.) rather than sucrose – also at 16% and 2% w/w. The experimental procedure was identical to Experiment 1A, with the exception that the pretraining phase was only 1 session in length due to the rats' previous experience in the testing chambers.

### ***Experiment 2***

#### *Subjects*

48 Lister-Hooded rats (Charles River, UK) were used. They were aged approximately 8 weeks on arrival and had free-feeding weights ranging from 249-287g. All rats were housed and fed under the same conditions as in Experiments 1A and 1B.

#### *Materials & Procedure*

Experiment 2 used the same general methods as Experiments 1A and 1B. All rats received training with flavour and audio CSs (one using sucrose and the other using maltodextrin as the US), and with the order of exposure to CS and US type counterbalanced across the experiment. Because only the flavour CS data is reported here – each animal is

reported only once, half with sucrose as the US (Experiment 2A) and half with maltodextrin (Experiment 2B).

### ***Data analysis***

Analysis focused on two main measures; total consumption and mean lick cluster size (defined as a group of licks separated by intervals of less than 0.5 seconds – a criterion determined by the originators of this method (Davis & Perez, 1993; Davis & Smith, 1992) and used in most prior lick analysis studies from our lab (Dwyer, 2009; Dwyer, Lydall, & Hayward, 2011)<sup>1</sup>. Data was analysed using mixed ANOVAs, with a within subject factor of CS (CS+ or CS-), between subject factors of study (Experiment 1 or Experiment 2) and US type (sucrose or maltodextrin), with  $\alpha = 0.05$ . Only consumption was analysed for the 2-bottle tests as the opportunity to swap between bottles can influence the pattern of licking behaviour.

## **Results**

### **Training**

Training data for consumption is seen in Table 2. Consumption of the 16% carbohydrate solutions mixed with the CS+ was far higher than that of the 2% carbohydrate solutions mixed with the CS-, along with the slightly larger consumption scores seen across Experiment 1 than Experiment 2. ANOVA confirmed this impression revealing main effects of CS [ $F(1, 76) = 682.5$ ,  $p < 0.001$ ,  $\eta^2 = 0.9$ ,  $MSE = 2463.443$ ] and study [ $F(1, 76) = 3334.2$ ,  $p < 0.001$ ,  $\eta^2 = 0.978$ ,  $MSE = 201.644$ ] were seen, along with interactions between CS and study [ $F(1, 76) = 11.58$ ,  $p = 0.001$ ,  $\eta^2 = 0.132$ ,  $MSE = 41.781$ ] and CS and US type [ $F(1, 76) = 16$ ,  $p < 0.001$ ,  $\eta^2 = 0.174$ ,  $MSE = 57.759$ ]. There was no effect of US type [ $F(1, 76) = 0.38$ ,  $p = 0.539$ ,  $\eta^2 = 0.005$ ,  $MSE = 2.126$ ], and no study by US type interaction [ $F(1, 76) =$

2.28,  $p = 0.135$ ,  $\eta^2 = 0.029$ ,  $MSE = 12.759$ ] or CS by US type by study interaction [ $F(1, 76) = 2.23$ ,  $p = 0.131$ ,  $\eta^2 = 0.03$ ,  $MSE = 8.405$ ].

The lick cluster training data (also seen in Table 2) also clearly demonstrated higher palatability of the 16% over 2% carbohydrate solutions. Again a significant effect of CS was seen [ $F(1, 76) = 349.1$ ,  $p < 0.001$ ,  $\eta^2 = 0.821$ ,  $MSE = 28005.321$ ], along with significant interactions between study and US type [ $F(1, 76) = 7.94$ ,  $p = 0.006$ ,  $\eta^2 = 0.095$ ,  $MSE = 1003.425$ ] and CS, US type and study [ $F(1, 76) = 17.32$ ,  $p < 0.001$ ,  $\eta^2 = 0.186$ ,  $MSE = 1389.088$ ]. No main effects of study [ $F(1, 76) = 0.987$ ,  $p = 0.324$ ,  $\eta^2 = 0.013$ ,  $MSE = 124.712$ ] or US type [ $F(1, 76) = 1.41$ ,  $p = 0.239$ ,  $\eta^2 = 0.018$ ,  $MSE = 178.225$ ] were noted, nor interactions between CS and study [ $F(1, 76) = 0.987$ ,  $p = 1.99$ ,  $\eta^2 = 0.026$ ,  $MSE = 159.744$ ] or CS and US type [ $F(1, 76) = 1.07$ ,  $p = 0.304$ ,  $\eta^2 = 0.014$ ,  $MSE = 85.822$ ].

---- Table 2 about here ----

## 2-Bottle Test Phase

The 2-bottle consumption data are presented in Figure 1, and clearly show that a strong preference for the CS+ over the CS- was evident in all experiments. ANOVA revealed a main effect of CS [ $F(1, 76) = 142.9$ ,  $p < 0.001$ ,  $\eta^2 = 0.653$ ,  $MSE = 1041.042$ ], confirming the overall preference for the CS+. There were also main effects of study [ $F(1, 76) = 14.175$ ,  $p < 0.001$ ,  $\eta^2 = 0.157$ ,  $MSE = 68.427$ ] and US type [ $F(1, 76) = 8.866$ ,  $p = 0.004$ ,  $\eta^2 = 0.104$ ,  $MSE = 42.799$ ]. There was a three-way interaction between CS, study and US type [ $F(1, 76) = 16.521$ ,  $p < 0.001$ ,  $\eta^2 = 0.179$ ,  $MSE = 120.346$ ], indicating the relative size of the preferences conditioned by sucrose and maltodextrin reversed between Experiment 1 to Experiment 2. There were no interactions between CS and study [ $F(1, 76) = 0.144$ ,  $p = 0.706$ ,  $\eta^2 = 0.002$ ,  $MSE = 1.047$ ] or CS and US type [ $F(1, 76) = 1.045$ ,  $p = 0.310$ ,  $\eta^2 = 0.014$ ,  $MSE = 7.615$ ]. Despite the three-way interaction, simple effects analysis showed

confirmed that the CS+ was preferentially consumed to the CS- for each subgroup;

Experiment 1A [ $F(1, 76) = 63.225, p < 0.001, \eta^2 = 0.454$ ], Experiment 1B [ $F(1, 76) = 10.94, p = 0.001, \eta^2 = 0.126$ ], Experiment 2A [ $F(1, 76) = 22.756, p < 0.001, \eta^2 = 0.23$ ], and Experiment 2B [ $F(1, 76) = 66.77, p < 0.001, \eta^2 = 0.468$ ].

---- **Figure 1 about here** ----

### 1-Bottle Test Phase

The lick cluster size data from the 1-bottle tests is shown in Figure 2. Inspection of the figure suggests that although the lick cluster sizes were generally higher for the CS+ than the CS-, the difference was relatively small overall and inconsistent across the different groups. An ANOVA confirms this impression, revealing a main effect of CS [ $F(1, 76) = 5.77, p = 0.019, \eta^2 = 0.071, \text{MSE} = 1117.075$ ], as well as main effects of study [ $F(1, 76) = 8.714, p = 0.004, \eta^2 = 0.103, \text{MSE} = 9492.3$ ], and US type [ $F(1, 76) = 21.107, p < 0.001, \eta^2 = 0.217, \text{MSE} = 22992.663$ ]. There was no interaction between CS and US type [ $F(1, 76) = 0.044, p = 0.835, \eta^2 = 0.001, \text{MSE} = 8.43$ ], or CS and study [ $F(1, 76) = 1.75, p = 0.19, \eta^2 = 0.023, \text{MSE} = 338.746$ ]. There was a significant three-way interaction between CS, study and US type [ $F(1, 76) = 4.943, p = 0.029, \eta^2 = 0.061, \text{MSE} = 956.993$ ]. This was the converse of that seen with the 2-bottle consumption data: with the size of the CS+ vs CS- difference higher for maltodextrin than sucrose in Experiments 1A/1B, but higher for sucrose than maltodextrin in Experiments 2A/2B. Simple effects analysis of the four subgroups considered alone showed a significant effect of CS only in Experiment 1B [ $F(1, 76) = 7.898, p = 0.006, \eta^2 = 0.094$ ] - not in Experiment 1A [ $F(1, 76) = 0.348, p = 0.557, \eta^2 = 0.005$ ], Experiment 2A [ $F(1, 76) = 2.992, p = 0.088, \eta^2 = 0.038$ ] or Experiment 2B [ $F(1, 76) = 0.273, p = 0.603, \eta^2 = 0.004$ ].

Table 3 shows the consumption data from the 1-bottle tests. There was no effect of CS on this consumption data [ $F(1, 76) = 0.407$ ,  $p = 0.526$ ,  $\eta^2 = 0.005$ ], which is to be expected due to both CSs being presented separately with 16% carbohydrate (see Dwyer 2008; Harris & Thein, 2005). Due to differences in consumption between studies there was an effect of study [ $F(1, 76) = 15.178$ ,  $p < 0.001$ ,  $\eta^2 = 0.166$ ] and a US type by study interaction [ $F(1, 76) = 22.513$ ,  $p < 0.001$ ,  $\eta^2 = 0.229$ ]. There was no effect of US type [ $F(1, 76) = 0.592$ ,  $p = 0.444$ ,  $\eta^2 = 0.008$ ], and no interaction between CS and US type [ $F(1, 76) = 2.263$ ,  $p = 0.137$ ,  $\eta^2 = 0.029$ ], CS and study [ $F(1, 76) = 1.332$ ,  $p = 0.252$ ,  $\eta^2 = 0.017$ ], or CS US type and study [ $F(1, 76) = 1.085$ ,  $p = 0.301$ ,  $\eta^2 = 0.014$ ].

---- Figure 2 & Table 3 about here ----

### Meta-analysis

While mean lick cluster size was, considered over all studies, higher for the CS+ than the CS- in the 1-bottle tests, the difference was relatively small and only inconsistently observed. To formalise these observations, we performed a meta-analysis across the current experiments and similar published reports of lick analysis in flavour preference learning in rats. Lick cluster data from both groups used in Experiment 2 of Dwyer (2008), from test session one of Dwyer et al. (2009) and the current experiments were therefore collated<sup>2</sup>, and the Exploratory Software for Confidence Intervals (ESCI) (Cumming, 2013) Excel package was used to calculate the combined effect size using a fixed effects model. The combined estimate of the conditioned difference in lick cluster size between the CS+ and the CS- at test was 4.73, 95% CI (2.22, 7.25) – corresponding to a Cohen's  $d_{av}$  (Lakens, 2013) of 0.16. In addition, for mice, consumption of CS flavours paired with sucrose has once been reported to produce both a conditioned increase in consumption and lick cluster size (Experiment 4 of

Austen et al., 2017), and twice to produce conditioned changes in consumption with no significant change in lick cluster size (Experiment 2 of Austen & Sanderson, 2016; Experiment 5 of Austen et al., 2017): the combined effect size of these mouse studies corresponds to a Cohen's  $d_{av}$  of 0.36.

To put these combined effect sizes in context, consider the remaining studies that have used other conditioning and measurement methods to assess hedonic change after flavour preference learning. Namely orofacial response measurement after either free consumption or oral infusion of the CS with glucose (Forestell & LoLordo, 2003), oral infusion of the CS followed by oral infusion of sucrose (Breslin et al., 1990), and both licking microstructure analysis and orofacial measurement after consumption or oral infusion of the CS was paired with intragastric nutrient delivery (Myers & Sclafani, 2001a, 2001b). Cohen's  $d_{av}$  values for the CS+ vs CS- difference for these studies were: 1.15 for Experiment 1 and 0.98 for Experiment 2 from Forestell & LoLordo (2003), 0.56 for appetitive taste reactivity and 0.51 for aversive taste reactivity (Breslin et al., 1990), 0.85 for Experiment 1 from the intragastric lick cluster test (Myers & Sclafani, 2001a), and 0.67 for Experiment 1A from the intragastric taste reactivity test (Myers & Sclafani, 2001b). Clearly, the effect sizes for conditioned changes in cue flavour palatability after preference conditioning are much larger in all these circumstances compared to the current combined effect sizes for conditioned changes in lick cluster size following oral consumption of the CS mixed with the reinforcing solution. It is unlikely that this be attributable to the overall size of the preference conditioning effect per-se, because the 2-bottle preference results reported here are within the same range as those from previous reports (and indeed, the largest change in lick cluster size here was in the group of animals with the lowest 2-bottle preference). It is also worth noting that the unconditioned effect size in response to different carbohydrate concentrations in

mean lick cluster size was 1.85. This suggests that the current assessment methods were more sensitive to conditioned than unconditioned hedonic effects.

## Discussion

To summarise, a total of four separate experimental groups consisting of two replications, each pairing a CS+ flavour with 16% sucrose or maltodextrin and a CS- flavour with 2% sucrose or maltodextrin, showed a clear increase in consumption of the CS+ flavour over the CS- in all 2-bottle tests – indicating a strong learned flavour preference for the CS+. However, licking microstructure analysis of the 1-bottle tests showed only a small overall increase in mean lick cluster size for the CS+ compared to the CS-. A meta-analysis, combining data from these four groups with results from the two previously published studies using a similar method, revealed a small Cohen's  $d_{av}$  effect size of 0.16. Thus, there is a learned effect of preference on mean lick cluster size, but it is a small one. An examination of published work using different methods (assessment using taste reactivity and/or training using intragastric nutrient infusion) to investigate hedonic change in flavour preference conditioning revealed that all of these had larger Cohen's  $d_{av}$  values ranging from 0.51 to 1.15. These effects are much larger than that found here with licking microstructure analysis and training with oral consumption of the CS and US mixed together. Thus, while all methods are consistent in indicating that flavour preference learning does produce a change in the hedonic properties of the CS+, the method chosen to investigate this clearly influences the apparent size of the effect.

Before discussing the potential differences between conditioning and assessment methods, it is worth considering other previous reports where successful flavour preference conditioning was observed in consumption without reliable conditioned changes in palatability. In rats, using mildly aversive CS flavours and intragastric glucose infusion did



not impair the size of conditioned changes in preference for consumption, but there was no significant increase in appetitive taste reactivity responses (or significant decrease in aversive responses) (Myers & Sclafani, 2003). That said, Breslin et al. (1990) did observe conditioned changes to both appetitive and aversive taste reactivity responses when oral infusion of mildly aversive CS flavours was followed by oral infusion of sucrose. One obvious possibility here is that the presence of a detectable palatability change following flavour preference conditioning may be influenced by the unconditioned reaction to the CS, because otherwise identical work with more neutral CS flavours and intragastric glucose infusion did produce clear evidence of conditioned changes in palatability (Myers & Sclafani, 2001b). In contrast, in mice, training through voluntary consumption of a CS flavour mixed with a concentrated sucrose US, followed by testing of consumption and lick analysis measures of palatability, one experiment showed a conditioned increase in palatability, and two did not, despite all showing conditioned changes in consumption (Austen & Sanderson, 2016; Austen et al., 2017); and the combined effect size was – like with rats – small but positive. Given that these mouse studies used comparable training and assessment methods to the ones we report here in rats, it suggests that it is the methods, rather than the species, which might be critical.

While it should be noted that some of the different training and test methods for investigating hedonic changes in flavour preference learning have been reported only once, and thus the precision in the relevant estimates might be low, it is still worth considering possible explanations for the heterogeneity in the effect sizes. Unlike taste reactivity testing, licking microstructure analysis relies on voluntary consumption, and thus may be more susceptible to ceiling and/or floor effects. Moreover, although no formal comparison the sensitivity of lick analysis and taste reactivity testing to hedonic changes has been made, it is possible that taste reactivity measures may simply be more sensitive. It is also possible that the way in which the CS and US are experienced throughout the training and test phases

could influence the results. With taste reactivity testing and intragastric nutrient infusion, rats were never given a combined solution of both CS and US – and so the effective salience of the CS might be higher if its flavour is not masked at all by the concurrent presence of a carbohydrate. Alternatively, there may be more generalisation decrement as the presence/absence of the US changes between training and test. To the extent that these factors do influence the detection of hedonic changes, then the methods used here – licking microstructure analysis and training/testing through voluntary consumption of the CS and US mixed together – may underestimate the true size of the underlying effect. It is also possible that the current training methods, in particular the concurrent mixture of CS and US, may limit the degree of conditioned palatability change directly. On the basis of the current results, it is not possible to distinguish between influences on the size of a conditioned palatability change or the detection of such an effect.

In conclusion, our current experiments are consistent with previous reports that pairing a flavour CS with nutrients results in a learned increase in lick cluster size. However, the current results and meta-analysis indicate that this effect is small (and closely related mouse experiments reveal similar effects), especially when considered against alternative methods for assessing hedonic reactions to flavours. This may be due a lack of sensitivity of the analysis of licking microstructure to detecting positive hedonic changes, or a lack of effectiveness when training with simultaneous consumption of the CS and US in a single solution. Thus, our results imply that future studies of hedonic changes in flavour preference learning may wish to consider focusing on alternative training (e.g. intragastric nutrient infusion or other means of separate CS/US presentation) and/or testing (e.g. taste reactivity testing). However, these alternative training methods have only been combined with palatability assessment relatively few times, and given the heterogeneity of the results across

different training and testing methods, the factors determining the presence and degree of hedonic change remain to be fully determined.

## Footnotes

<sup>1</sup>Mean inter-lick interval (ILI) and volume consumed per 1000 licks (volume/1000 licks) can also be determined. These control variables indicate whether there are differences in the nature of the drinking behaviour that could confound interpretation of the lick cluster size. However, there were no significant differences between conditions on these variables and so they have not been reported in detail.

<sup>2</sup>Although the data reported in Dwyer et al. (2009) used 1s as the criterion for defining lick clusters, we re-analysed that data with 0.5s as the criterion to ensure a common measure was used across the meta-analysis.

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## Figure Captions

*Figure 1:* Bars show mean consumption of the CS+ and CS- for Experiments 1A, 1B and 2 in the 2-bottle test phase as a function of experiment and US type. Error bars represent the standard error of the mean (SEM). Numbers above each set of bars represent the preference ratio (with SEM) for the CS+ over total consumption.

*Figure 2:* Bars show mean lick cluster size for the CS+ and CS- for Experiments 1A, 1B and 2 in the 2-bottle test phase as a function of experiment and US type. Error bars represent the standard error of the mean (SEM).

Figure 1:

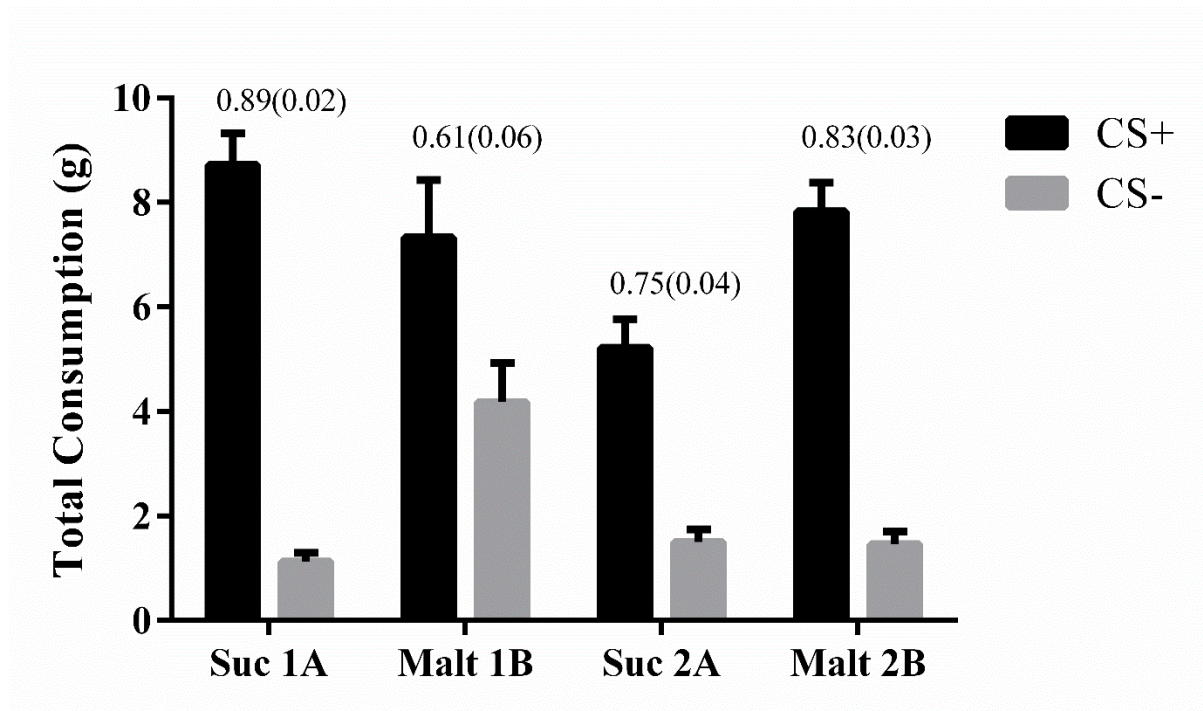


Figure 2:

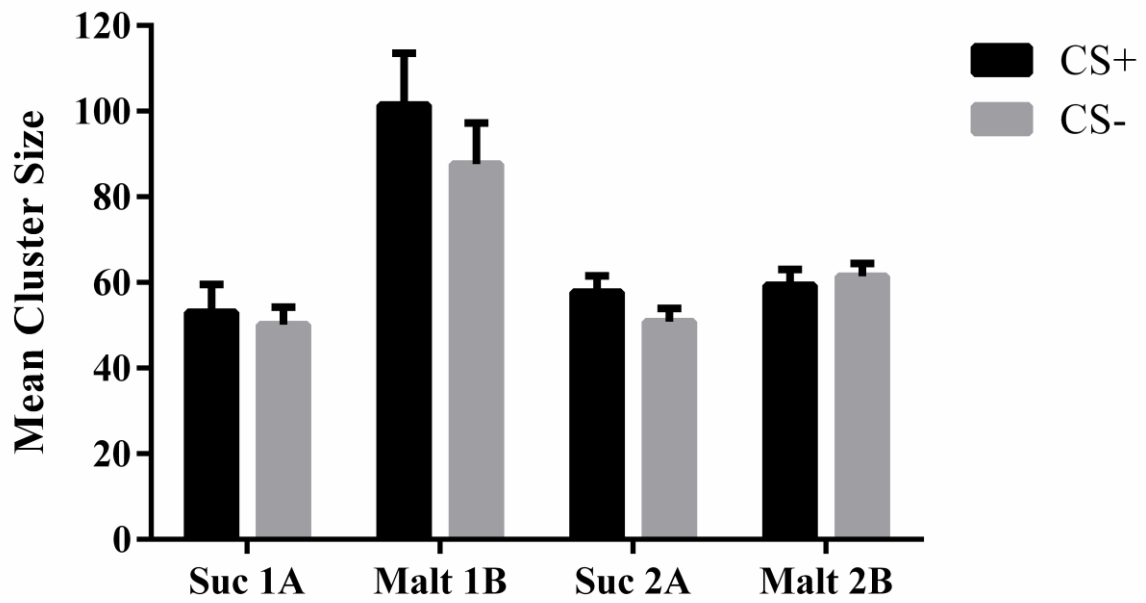




Table 1

## General Design

Experiment	US	Training (repeated 4 times)		1-bottle test	2-bottle test
1A	Sucrose	CS+ & 16%	CS- & 2%	CS+ / CS- (separate days)	CS+ vs CS-
1B	Maltodextrin	CS+ & 16%	CS- & 2%	CS+ / CS- (separate days)	CS+ vs CS-
2A	Sucrose	CS+ & 16 %	CS- & 2 %	CS+ / CS- (separate days)	CS+ vs CS-
2B	Maltodextrin	CS+ & 16 %	CS- & 2 %	CS+ / CS- (separate days)	CS+ vs CS-

Note: All sessions were 20 minutes long. During training, rats were presented with the CS+ and CS- (each mixed with the relevant carbohydrate solution) on separate days and so the training phase required 8 sessions total. The 1-bottle testing phase took place over two days (with the order of CS+ and CS- testing counterbalanced).

Table 2:

Mean consumption and lick cluster scores across training for all experiments

Experiment	US	Consumption	Consumption	Lick Clusters	Lick Clusters
		CS+	CS-	CS+	CS-
1A	Sucrose	15.6 (0.60)	7.9 (0.77)	42.6 (3.77)	21 (1.11)
1B	Maltodextrin	15.7 (0.44)	9.5 (0.68)	57.3 (4.10)	20.8 (1.41)
2	Sucrose	15.4 (0.48)	4.7 (0.33)	49.9 (2.77)	20.4 (1.08)
2	Maltodextrin	13.4 (0.38)	6 (0.29)	42.4 (1.87)	21.9 (1.10)

Note: Table shows amount consumed in grams for consumption scores and mean lick cluster size in lick cluster columns. Numbers in parentheses are the SEMs.

Table 3:

Consumption in 1-bottle test

Experiment	CS+	CS-
1A	17.7 (0.75)	18.1 (0.52)
1B	22.0 (0.70)	19.9 (0.68)
2A	18.3 (1.06)	19.2 (0.89)
2B	17.2 (0.64)	16.4 (0.89)

Note: Table shows amount consumed in grams for the CS+ and CS- for Experiments 1A, 1B and 2. Number in parentheses is the SEM.

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